World Academy of Science, Engineering and Technology
International Journal of Medical and Health Sciences
Vol:11, No:4, 2017

Ellagic Acid Enhanced Apoptotic Radiosensitivity via G1 Cell Cycle Arrest and γ-H2AX Foci Formation in HeLa Cells *in vitro*

V. R. Ahire, A. Kumar, B. N. Pandey, K. P. Mishra, G. R. Kulkarni

***Abstract*—**Radiation therapy is an effective vital strategy used globally in the treatment of cervical cancer. However, radiation efficacy principally depends on the radiosensitivity of the tumor, and not all patient exhibit significant response to irradiation. A radiosensitive tumor is easier to cure than a radioresistant tumor which later advances to local recurrence and metastasis. Herbal polyphenols are gaining attention for exhibiting radiosensitization through various signaling. Current work focuses to study the radiosensitization effect of ellagic acid (EA), on HeLa cells. EA intermediated radiosensitization of HeLa cells was due to the induction γ-H2AX foci formation, G1 phase cell cycle arrest, and loss of reproductive potential, growth inhibition, drop in the mitochondrial membrane potential and protein expression studies that eventually induced apoptosis. Irradiation of HeLa in presence of EA (10 μM) to doses of 2 and 4 Gy γ-radiation produced marked tumor cytotoxicity. EA also demonstrated radio-protective effect on normal cell, NIH3T3 and aided recovery from the radiation damage. Our results advocate EA to be an effective adjuvant for improving cancer radiotherapy as it displays striking tumor cytotoxicity and reduced normal cell damage instigated by irradiation.

***Keywords*—**Apoptotic radiosensitivity, ellagic acid, mitochondrial potential, cell-cycle arrest.

I. Introduction

CERVICAL cancer remains the second major root of cancer mortality in women with more than 270 000 deaths per year worldwide [1]. Radiotherapy continues to play a significant role in definitive as well as adjuvant therapy of cancer patients. The success of cancer therapy lies in enhanced toxicity to cancer cells by external agents, namely, radiation, drug or their combinations. Cancer cells resist to therapeutic agents by shutting off the pathways for induction of apoptosis. Therefore, strategy of effective killing cancer cells rests in triggering of apoptotic process. Signaling mechanisms involving Bcl-2 family members play decisive role in determining the fate of the cell to either survive or undergo apoptosis. Cellular response to radiation or drug is regulated by apoptogenic factors released from the mitochondria and which are associated with death proteases such as caspases [2]. Amongst the cysteine proteases which play essential role in the induction of apoptosis, activation of caspase-3 is a central downstream event in cell death process [3], [4].

It is commonly observed in clinic that local recurrence and radio-resistance pose challenge following radiotherapy [5] which adversely affects the treatment success. Some of the key causes of radioresistance are hypoxia in tumor microenvironment [6], [7] and efficient restoration of DNA damage in tumor cells [8]. There is increasing evidence that Her-2, EGFR, AKT, Cox-2 regulate cervical cancer radio-resistance in diverse ways [9]-[14]. However, mechanisms of cervical cancer radioresistance are still largely unclear. Therefore, research is warranted to discover novel apoptosis-inducing compounds that may prove potential anti-tumor drug.

Dietary components play an essential role in preventing from various tumors like breast, colon, ovary, cervical, oral, head, neck, and so on. Constituents, such as green tea, curcumin, garlic, ginger, chilies, soya, tomatoes, onion, and cruciferous vegetables are generally considered potent natural products to prevent physiological alterations, frenzied proliferation, and inflammatory reactions that are believed to initiate the tumorigenesis. Plant derived compounds are pharmacologically safe [15] and they are capable of delaying the initiation of carcinogenesis. In recent years, it has become known that they enhance the tumor radiation damage [16]-[20]. These results generate possibility of developing novel treatment approach which is not achievable by chemotherapy or radiotherapy alone. The present study was therefore undertaken to examine the potential of EA for producing enhanced killing of HeLa tumor cell line in combination with gamma radiation in vitro. Results are reported on the mechanism of EA mediated radiosensitization of tumor cells evaluated in terms of cellular viability, clonogenic assay, cell cycle analysis, induction of H2AX, and loss of mitochondrial potential.

II. Materials and Methods

A. Cell Culture, EA Treatment, and Cell Irradiation

HeLa, human cervical cancer cell line, was purchased from National Center for Cell Sciences, Pune, India and were incubated in a humidified atmosphere at 37 °C in 5% CO_2 environment. It was cultured in Dulbecco's Modified Eagle's Medium (DMEM) purchased from Gibco Co., USA. The growth media was supplemented with 10% FBS and antibiotics like 200 μg/ml streptomycin and 100 units /ml penicillin (Gibco Co.)

K. P. Mishra is with Foundation for Education and Research, India and BM International Research Centre, Mumbai, India (e-mail: mishra_kaushala@rediffmail.com).

V. R. Ahire and G. R. Kulkarni are with School of Basic Medical Science; University of Pune, Pune-41100, India (e-mail: vidhula.ahire@gmail.com, grk@physics.unipune.ac.in).

A. Kumar and B. N. Pandey are with Bhabha Atomic Research Centre, Mumbai, India (e-mail: amithere4@rediffmail.com, bnp@barc.gov.in).

EA (E-2250) was purchased from Sigma. Stock solutions of EA which were prepared in DMSO were protected from light and stored at 4 °C. Dilutions were prepared freshly in the respective media with FCS, prior to experiments. Irradiation was carried out at room temperature using Co-60 γ-rays at a dose rate of 1 Gy/ min (Bhabhatron- II, Panacea Technologies, and Bangalore, India). For combination treatments, EA was added to the cultures 3h prior to radiation treatment.

B. Cell Proliferation and Viability by Trypan Blue

Briefly, the dose-dependent effects of increasing dose of radiation (0, 2, 4 Gy) and EA (10 μM) on the viability of 1 × 105 cells/mL of HeLa grown in DMEM supplemented with 10% FCS was determined by trypan blue dye exclusion assay. Cells were treated with EA (10 μM) 3h prior to radiation. Treatment was given for 24, 48, or 72h at 37 °C.

At the end of each treatment, viability of the cells was characterized by trypan blue dye exclusion assay (final volume of the dye 0.04 % w/v) using a hemocytometer under an inverted microscope. Four independent experiments were performed, and the mean with standard deviations was calculated.

C. Clonogenic Assay

Log-phase cells were plated into 6-well plates at initial cell density varying from 50-800 cells/ well and left treated or untreated with EA (10μM) and/ or radiation (0-6 Gy). Medium was replaced with fresh DMEM after 48 and 72h and was maintained for two weeks in order to allow cells to form colonies. Approximately after two weeks of culture, the plates were fixed with ethanol and stained with 0.5% crystal violet. The number of colonies per plate was assessed and then expressed as in terms of survival fraction (Survival Fraction= Colonies counted/ (cells seeded*PE/100)).

D. Morphological Studies

Cells were grown in petri plates until they were 60% confluent. They were treated with irradiation (2 Gy) or EA (10 μM) or in combination for 48h. To understand the cell morphology and apoptotic bodies, phase contrast images were taken at the end of 48h.

E. ϒ-H2AX

HeLa cells were cultured on coverslips and treated with EA prior to 2 and 4 Gy of gamma irradiation. Cells were allowed to recover for 30 min, 3h and 24h, fixed with paraformaldehyde, permeabilized with Igepal, and stained with anti-Y-H2AX antibody. Staining was visualized using FITC-conjugated anti-rabbit AlexaFLour-488 secondary antibody (Invitrogen, USA). Coverslips were mounted with antifade mounting media containing DAPI to counter the cellular nuclei. γ- H2AX foci were manually scored by using a confocal microscope (510META, Carl Zeiss, Germany) with a 63X objective and the average number of foci per cell was calculated from a minimum of 250 cells per dose/time point.

F. FACS of PI Staining

Briefly 1 x 106 cells were treated with radiation, EA alone or in combination for 24h, 48h, and 72h and trypsinized. The suspended cells were washed with PBS, centrifuged, and re-suspended in 300 μl of PBS. Samples were fixed by adding 70% (v/v) ice cold ethanol to the cell suspension keeping the tubes on ice. After washing twice with PBS, cells were incubated at 37 °C in the presence of 1 mg/ml RNase A for 1h. Finally, DNA was stained with propidium iodide (50 μg/ ml). Analysis was performed by using Partec Cyflow cytometer.

G. Mitochondrial Changes

In order to evaluate the effect of radiation and EA on the mitochondria, cells were grown on the coverslips as previously described and treated with or without radiation (2 Gy) and EA (10 μM). After 48h, cells were fixed with 4% paraformaldehyde for 15 min and then incubated in a medium containing 40 nM of MitoFlour for 20 min at culture conditions. Coverslips were then mounted with anti-fade Mounting Medium containing DAPI (ProLong Antifade reagent containing DAPI 50 nM, Invitrogen) and sealed with nailpaint. Cells were imaged by using Carl Ziess Confocal Microscope with a 63X objective.

H. Protein Expression Studies

HeLa cells were treated with EA (10 μM), radiation (2, 4 Gy) alone or in combination. The harvested cells were pelleted by centrifugation, washed with PBS, and lysed cold lysis buffer containing protease inhibitor cocktail for 15-20 mins on ice with intermittent mixing. The protein quantification was done with a protein assay kit (Bio-Rad Laboratories). A 50-μg aliquot of the total protein was separated by SDS/PAGE and transferred to PVDF membranes (Roche, Mannheim, Germany) for immunological detection of the proteins. The blots were probed using antibodies against Bad, Bcl-2, PARP, Caspase3 (Cell Signaling Technology), and β-actin (CST) according to the manufacturer's instructions. The immunoreactions were visualized by using ECL-Plus detection system LumilightPlus, Roche, USA). The membranes were then imaged and autoradiograped by using X-ray film.

I. Proliferation Studies on Trypan Blue Dye Exclusion Assay

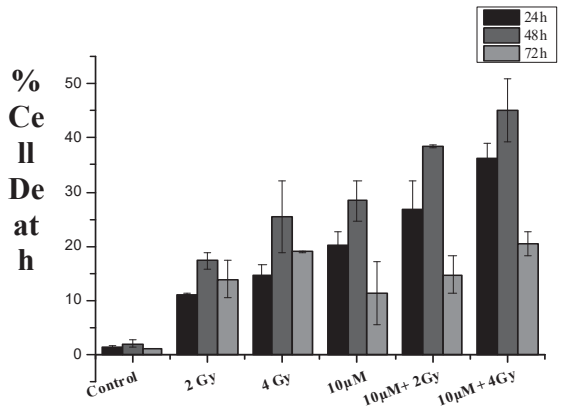
Briefly, 1 × 105 cells were grown in DMEM supplemented with 10 % FCS. The dose-dependent effects of increasing dose of radiation (0, 2, 4, 6 Gy) on the viability of NIH3T3 cells were determined by trypan blue dye exclusion assay.

Treatment was given for 48h at 37 °C. At the end of the treatment, viability of the cells was calculated by trypan blue dye exclusion method (final volume of the dye 0.04% w/v) using a Hemocytometer under an inverted microscope. A total of four independent experiments were executed and the mean with standard deviations were determined.

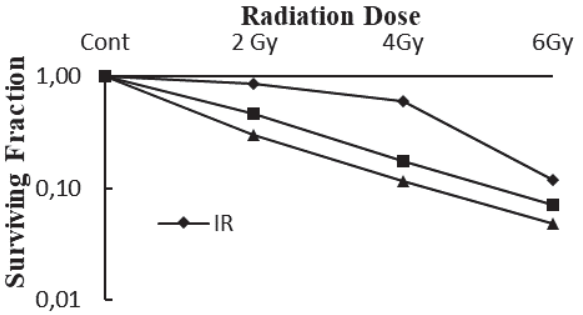
J. Statistical Analysis

Data represented in the study are mean ± SE of four replication experiments. Statistical significance was tested by comparisons made using students t-test. The difference was considered significant for p-values <0.05 were considered

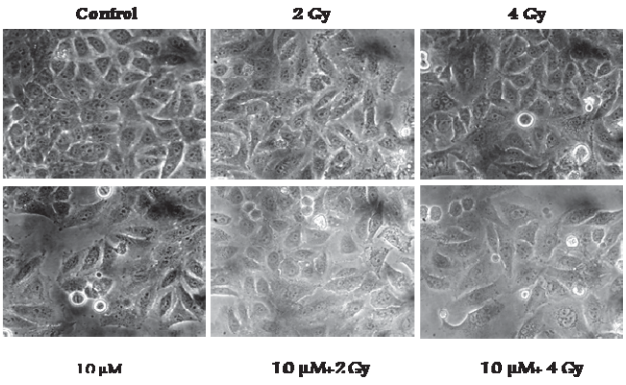
significant. Data analysis was performed by using Origin 9.1.



(a)



(b)



(c)

Fig. 1 Role of EA enhanced and mediated radiation induced cytotoxicity in HeLa, human cervical cancer cells: (a) Cell Death in HeLa cells followed by treatment of EA (10μM), radiation (2, 4 Gy) alone or in combination at 24, 48, and 72h was measured by trypan blue exclusion assay as described in material and methods; (b) Cell reproductive potential after the treatment of EA in combination with radiation was determined by colony forming assay; (c) Phase contrast images of morphological changes and apoptotic cells

III. Results

A. Effect of EA and IR on Cell Death

Fig. 1 (a) depicts the % of cell death as a function of EA treatment time for 24, 48, and 72 h. Treatment of cells with EA (10 μM) showed significant cell death at 48h as compared to 24 and 72h. It can be seen that following irradiation at 2 and 4 Gy after incubation for 48h, per cent cell death was 17.2 and 25.5 % respectively. Treatment with EA alone showed 28.4% cell death. An additive effect ($p<0.05$) of 45% cell killing was seen in the EA and radiation combined treatment (10 μM EA+ 4 Gy).

B. Effect of EA and IR on Colony Forming

Graphical representation in Fig. 1 (b) clearly showed that EA treatment caused a dose dependent decrease in the colony forming ability of HeLa cell lines. The slopes of survival curves of HeLa for combined EA and IR treated cells were steeper than that of IR alone at 2, 4, and 6 Gy radiation doses that indicated combined treatment of EA (10 μM) and IR significantly ($P<0.05$) reduced the capacity of HeLa cells to form colonies compared with individual treatments.

C. Apoptotic Effect of EA on Irradiated HeLa Cells

The morphology of the cell nucleus is shown in Fig. 1 (c). The treatment of HeLa cells with EA for 48h resulted in notable cell shrinkage, cell detachment, and loss of the originally confluent monolayer of cells.

D. Effect of EA on γ-H2AX in Radiation Treated Cells

It can be seen from Fig. 2 (a) that maximum number of γ-H2AX foci was found at 15 mins after irradiation with ~ 22-27 foci/cell for 2 Gy exposure and ~33-38 foci/cell in EA+2Gy IR. At 3h, 28-33 foci/cell and at 24h, 23-27 foci/ cell were observed. Treatment with EA alone did not form any double strand breaks, and negligible foci were found in EA treated samples.

E. Effect of EA on Cell Cycle of Irradiated Cells

Experiments were conducted to measure changes in cell cycle of HeLa cells after treatment either alone or in combination. Results showed that after 48h of radiation (2, 4 Gy), ~ 35-40% cells accumulated in the G1-S phase of the cell cycle (Fig. 2 (b)). These cells appeared to be blocked in G1 phase for > 48h after irradiation because high G1-low S phase percentages were maintained. When treated with EA alone, 45% of cells were arrested in the G1-S phase. HeLa cells treated with EA (10 μM) in combination with radiation (2, 4 Gy) showed that significant accumulation of cells was found to be in the apoptotic phase which was significantly higher in the combined treatment compared to that of with only EA or IR alone.

It is seen from Fig. 2 (b) that the increase in the percentage of apoptosis in the Sub G1 phase from 1.23 % in control; 9.2% in EA alone; 4.8% and 7.9 % in 2 and 4 Gy of radiation treatment alone and 20.12 and 35.38% in combined treatment of EA+ 2Gy and EA+ 4 Gy treatment, respectively.

F. Effect of EA and Radiation on the Mitochondria

The histogram panel (Fig. 3 (a)) demonstrates that EA treatment caused significant loss in the mitochondrial membrane potential. A 2.6-fold decrease was observed in the combined treatment of EA and IR, whereas 1.7 and 1.3-fold

decrease was observed in EA and IR treatment, respectively.

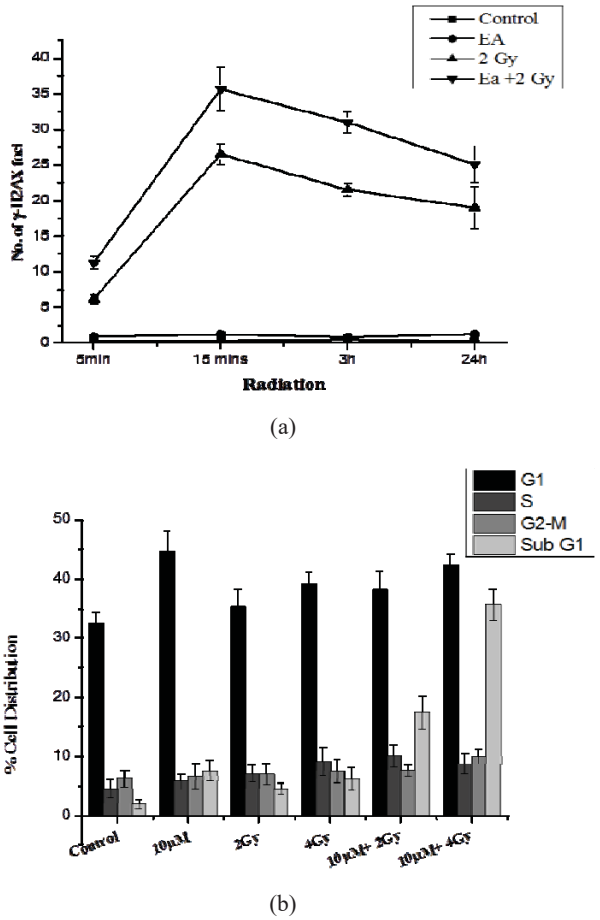


Fig. 2 EA-mediated enhancement in radiation cytotoxicity involves higher DNA damage and cell cycle arrest. (A) γ-H2AX foci formation after 3h of EA treatment followed by 2 Gy radiation. The foci kinetics was measured at 5 mins, 15 min and 3h and 24h time points. (B) A representative cell cycle analysis performed by flow cytometer of EA and/ or radiation treated HeLa cells is shown

G. Subcellular Fractionation and Western Blot Analysis

We examined whether EA induced apoptosis was caspase dependent. The treatment of HeLa with EA and/ or IR lead to up-regulation of BAD (23 kDa), a pro-apoptotic protein induced apoptosis by release of cytochrome C and Caspase 3. BAD is important regulator of cell death machinery. Bcl-2(28 kDa), anti- apoptotic protein was significantly down-regulated which otherwise interfered with the activation of prop-apoptotic proteins and prevents cell death. Our western blot data reveal that EA and/or IR lead to the activation of procaspase-3 by cleaving it to a functional Caspase 3 (37 kDa). PARP (116 kDa), being the substrate of Caspase 3 is cleaved, and hence, it cannot repair DNA DSB pushing the cell to apoptosis (Fig. 3 (b)).

H. Effect of EA and Radiation on NIH3T3 Cells

Fig. 4 (a) gives the % of cell death after EA, radiation and combination treatment of EA and radiation after 24, 48, and 72h. At 24 h the cell death was 7, 12, 18% for 2, 4, 6 Gy of radiation treatment alone. At 48h cell, death was found to be 16, 22, 30% 18% for 2, 4, 6 Gy of radiation treatment.

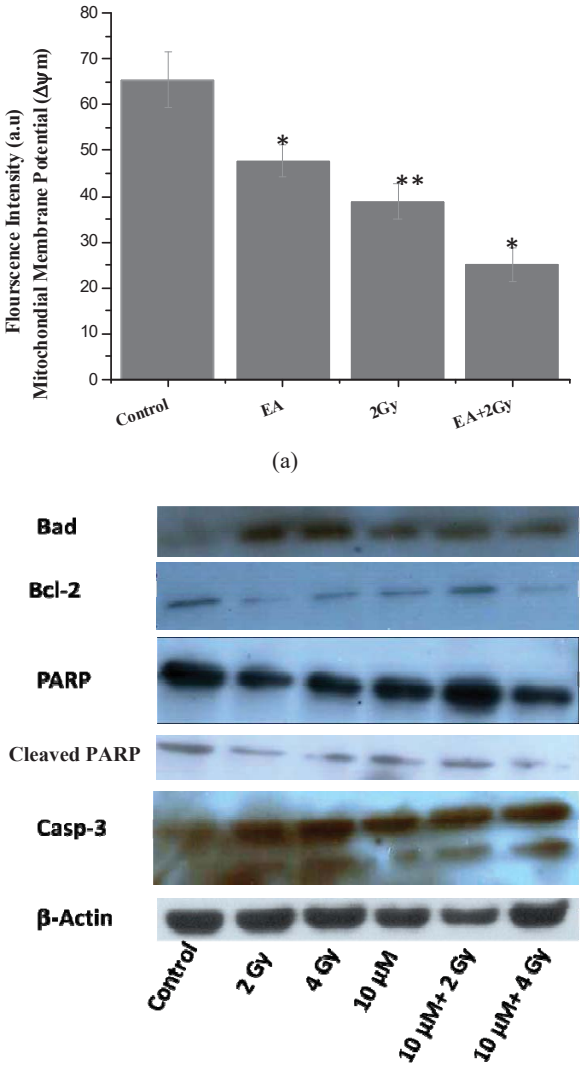
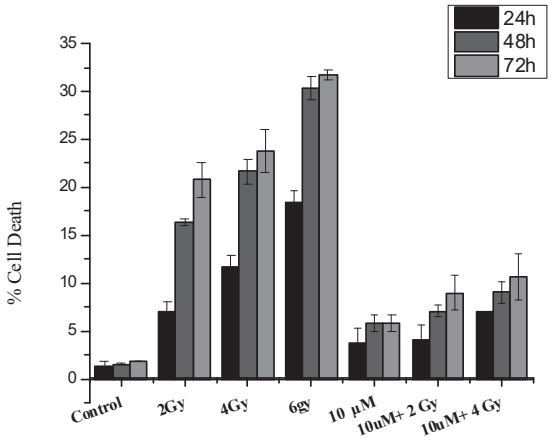


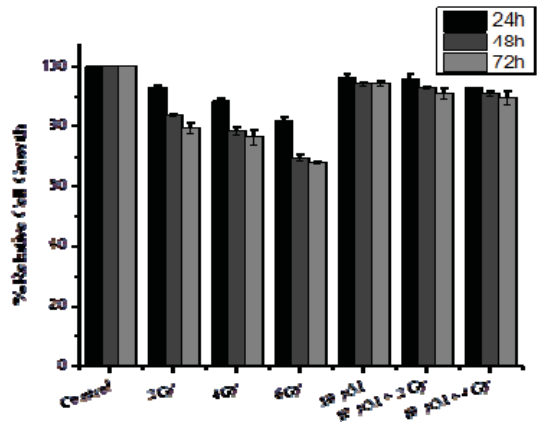
Fig. 3 EA enhanced radiation cytotoxicity via mitochondrial pathway of apoptosis. (A) Alterations in the mitochondrial trans-membrane potential (Δψm) of HeLa cells in response to EA treatment prior to 2 Gy radiation. The histogram clearly shows the drop in the MMP, whereas enhanced drop is observed in the combinatorial treatment of EA and IR as compared to that of individual treatment of EA and IR. Data are represented as a mean + SD from four independent experiments and are statistically significant at $p<0.05$ as compared to controls by student t-test. (B) Expression levels of proteins were analyzed by western blotting after treatment of EA and radiation. Mitochondrial membrane being a major site of Bcl-2 activity is altered due to induced expression of BAD. And release of cytochrome C activates the downstream caspase 3 which utilizes PARP as its substrate and cleaves it. The cells are pushed towards apoptosis as the DNA insults in the cell remain unrepaired, β- actin was used as a loading control. Data are representative of three independent experiments

When NIH3T3 cells were pretreated with EA (10 μM), it

showed significant reduction in the cell death at 24, 48, and 72 h. At 24h, pretreatment with EA prior to 2 and 4 Gy of radiation exhibited 4 and 7% cell death, respectively. At 48h, deaths reduced from 16 and 22 % to 7 and 9%, respectively, for 2 and 4 Gy treatment. Fig. 4 (b) shows the % relative cell growth after EA, radiation and combination treatment of EA and radiation for 24, 48, and 72h. The pretreatment with EA prior to radiation increased the % relative cell growth from 93 and 88% to 96 and 93%, respectively for 2 and 4 Gy at 24h. At 48h, there was a significant increase found from 84 and 78% to 93 and 92% for 2 and 4 Gy treatment.



(a)



(b)

Fig. 4 Radioprotective effect of EA on NIH3T3 cells in vitro (a) Cell death kinetics (b) Percentage relative cell growth in NIH3T3 cells followed by combined treatment of EA (10 µM) and irradiation (2, 4 Gy) at 24, 48, and 72h. Data are represented as a mean+SD from four independent experiments as calculated from software Origin 9. The experiments were performed in triplicates and were statistically significant at $p<0.05$ as compared to their control. (c) Morphology of NIH3T3 cells treated with EA for 24, 48, and 72h

IV. Discussion

One of the challenges of cancer chemo-radiotherapy is to abate the noxious effect of radiation on normal cells by reducing the radiation dose. This can be accomplished by using an adjuvant which enhances the radiation toxicity to tumor cells but at the same time spares normal cells. Since radiotherapy fails in the later stages of cancer due to acquiring radioresistance, it is imperative in radiobiology to upsurge the oxidative destruction of the tumor cells by using tumor-selective cytotoxic enhancers.

Ellagic acid is one such dietary polyphenolic drug that can elicit cell responses by activating various cellular molecular events. In the current study, we have investigated the role of EA mediated γ irradiation induced apoptosis in HeLa. Our results have shown that EA augments the growth inhibition and cell death of HeLa cells exposed to γ irradiation. Our experimental results have shown greater proportion of cells accumulated in the G1-S phase of the cell cycle indicating an arrest at G1 phase. Ionizing radiation induces DNA breaks (DSB) in cells that then trigger responses by the recruitment of DNA-repair proteins to γ-H2AX foci at locations of DNA damage and the stimulation of checkpoint proteins which arrest cell cycle advancement. As there was increased DNA damage in cells, reduced ability to repair DNA damage, and/or prolonged checkpoint activation EA caused cells to suffer G1 cell cycle arrest and eventually apoptosis. The loss of reproductive integrity and the inability to proliferate indefinitely after the combinatorial treatment of EA and IR were observed. EA facilitated he sensitization of HeLa cells and induced reproductive cell death in 48h. Even after supplementing fresh medium to the treated cells, they could not regain their reproductive potential.

Our DNA repair kinetics study of cells exposed to EA or radiation or combination of EA with IR, was done by assessing the response of γ-H2AX, a marker of DNA double strand breaks. The most dangerous type of lesion in the DNA is the double strand breaks (DSB), i.e. a complete break of the DNA double helix. Following radiation exposure, histone H2AX gets rapidly phosphorylated (within seconds) by the ATM and/or DNA-PK kinases at DNA DSB sites, reaching a peak of H2AX phosphorylation at around 15 minutes after radiation exposure. Although disappearance of these foci is associated with DNA damage repair, the damage is too severe to be repaired and consequently there were observed several foci even after 24h, indicating cell death.

There were alterations in the nuclear, morphological, and early apoptosis event, characterized by convoluted nuclei with cavitation and clumps of chromatin abutting to inner regions of the nuclear envelope between the nuclear pores. These pre-apoptotic modifications in the nucleus precede the externalization of phosphatidyl serine, condensation of chromatin and laddering of DNA, and can be separated from the formation of high molecular weight DNA fragments and cell shrinkage. Being vital organelle mitochondria is also adversely affected. Role of mitochondria in controlling of apoptosis has been well known in many signaling pathways. It controls and initiates apoptosis via loss of mitochondrial membrane potential ($\Delta\psi m$) by the discharge of apoptogenic factors from the intermembrane space into the cytosol. Hence, higher decrease in the mitochondrial membrane potential (MMP) was observed in HeLa cells that were sensitized to EA before irradiation. In this work, we show that, at the

commencement of apoptosis, alterations in MMP lead to cytochrome c release. Hence, diffused green fluorescence reveals the loss of mitochondrial potential.

It is known that the apoptosis death signal is determined by the ratio between pro- and anti-apoptotic proteins. Critical factors of EA prompted apoptosis including caspase activation and mitochondrial membrane potential loss with associated release of apoptogenic factors were analyzed. BAD (18.4 kDa) is phosphorylated and sequestered in the cytoplasm by the adapter protein, 14-3-3. When levels of free Bad in cytoplasm increase, Bcl-2 binds to Bad and discharges Bax and BAK which can then attach to the mitochondrial membrane and causes the release of cytochrome c dropping the mitochondrial membrane potential. This is followed by caspase activation, another hallmark of apoptosis. Caspase 3 leads to downstream break down of different cytoplasmic or nuclear substrates including PARP. These downstream cleavage events mark the morphological characteristics of apoptotic cell death. Known for being key molecules in cell death including drug induced apoptosis, triggering the activation of Caspase 3 seems to be a determinant of resistance or sensitivity to cytotoxic therapies. Because EA was able to augment the pro-apoptotic proteins, the HeLa cells proceeded to apoptosis through a caspase dependent pathway. One of the substrate of caspase is PARP. Activation of PARP is an instant cellular reaction to chemical, metabolic, or radiation-associated DNA SSB damage. On detecting a SSB, PARP binds to DNA and initiates synthesizing of poly (ADP-ribose) chain as an indication for DNA-repairing enzymes. Because caspase cleaves PARP, the DNA DSB cannot be repaired, and the cell dies. These data suggest that EA induced the increase in the pro-apoptotic protein and decreased the anti-apoptotic proteins through an intrinsic mitochondrial and caspase dependent apoptotic pathway. The combinatorial treatment of EA and radiation not only protected NIH3T3 cells from radiation damage but also helped them to repair the damage and survive healthily.

In conclusion, EA is found capable of inhibiting growth and enhancing the radiosensitivity of human cervical cancer cells which was mediated by G1 arrest, drop in mitochondrial membrane potential, down-regulation of anti-apoptotic factors, up-regulation of pro-apoptotic and critical apoptogenic factors like Caspase 3 and PARP.

ACKNOWLEDGMENT

We thank UGC; Government of India for the grant of Rajiv Gandhi Senior Research Fellowship (SRF) to Ms. Vidhula Ahire. We would also thank the authorities of Bhabha Atomic Research Centre for permitting the usage of facilities to conduct the above-mentioned work. Our special thanks to Prayag Amin, Sanjay Shinde, Hansa Yadav, Manzoor Ali and Vasumathy for providing technical assistance.

REFERENCES

[1] Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM: "Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008". Int J Cancer 2008, 2010 (127):2893-2917.

[2] Oltval ZN, Milliman CL, Korsmeyer SJ. " Bcl-2 heterodimerizes in vivo with a conserved homolog, bax, that accelerates programmed cell death". Cell 1993, 74:609-619.

[3] Earnshaw WC, Martins LM, Kaufmann SH. "Mammalian caspases: Structure, activation, substrates, and functions during apoptosis". Annual Review of Biochemistry 1999, 68:383-424.

[4] Shah S, Gapor A, Sylvester PW. " Role of caspase-8 activation in mediating vitamin E-induced apoptosis in murine mammary cancer cells". Nutrition and Cancer 2003, 45:236-246.

[5] Huang EY, Wang CJ, Chen HC, Fang FM, Huang YJ, Wang CY, Hsu HC. "Multivariate analysis of para-aortic lymph node recurrence after definitive radiotherapy for stage IB-IVA squamous cell carcinoma of uterine cervix".Int J Radiat Oncol Biol Phys 2008, 72:834-842.

[6] Hockel M, Schlenger K, Aral B, Mitze M, Schaffer U, Vaupel P. "Association between tumor hypoxia and malignant progression in advanced cancer of the uterine cervix". Cancer Res 1996, 56:4509-4515

[7] Rofstad EK, Sundfør K, Lyng H, Tropé CG. "Hypoxia-induced treatment failure in advanced squamous cell carcinoma of the uterine cervix is primarily due to hypoxia-induced radiation resistance rather than hypoxia-induced metastasis". Br J Cancer 2000, 83:354-359.

[8] Parliament MB, Murray D. "Single nucleotide polymorphisms of DNA repair genes as predictors of radioresponse". Semin Radiat Oncol 2010, 20:232-240.

[9] Hertlein L, Lenhard M, Kirschenhofer A, Kahlert S, Mayr D, Burges A, Friese K. "Cetuximab monotherapy in advanced cervical cancer: a retrospectivestudy with five patients". Arch Gynecol Obstet 2011, 283:109-113.

[10] Kurtz JE, Hardy-Bessard AC, Deslandres M, Lavau-Denes S, Largillier R, Roemer-Becuwe C, Weber B, Guillemet C, Paraiso D, Pujade-Lauraine E. "Cetuximab, topotecan and cisplatin for the treatment of advanced cervical cancer: a phase II GINECO trial". Gynecol Oncol 2009,113:16-20.

[11] Gaffney DK, Winter K, Dicker AP, Miller B, Eifel PJ, Ryu J, Avizonis V, Fromm M, Small W, Greven K. "Efficacy and patterns of failure for locally advanced cancer of the cervix treated with celebrex (celecoxib) and chemoradiotherapy in RTOG 0128". Int J Radiat Oncol Biol Phys 2007, 69:111-117.

[12] Herrera FG, Chan P, Doll C, Milosevic M, Oza A, Syed A, Pintilie M, Levin W, Manchul L, Fyles A. "A prospective phase I-II trial of the cyclooxygenase-2 inhibitor celecoxib in patients with carcinoma of the cervix with biomarker assessment of the tumor microenvironment". Int J Radiat Oncol Biol Phys 2007, 67:97-103

[13] Kim TJ, Lee JW, Song SY, Choi JJ, Choi CH, Kim BG, Lee JH, Bae DS. "Increased expression of pAKT is associated with radiation resistance in cervical cancer".Br J Cancer 2006, 94:1678-1682.

[14] Lee CM, Shrieve DC, Zempolich KA, Lee RJ, Hammond E, Handrahan DL, Gaffney DK. "Correlation between human epidermal growth factor receptor family (EGFR, HER2, HER3, HER4), phosphorylated Akt (P-Akt), and clinical outcomes after radiation therapy in carcinoma of the cervix". Gynecol Oncol 2005, 99:415-421.

[15] Bharat B Aggarwal, Haruyo Ichikawa, Prachi Garodia, Priya Weerasinghe,Gautam Sethi, Indra D Bhatt et.al. "From traditional Ayurvedic medicine to modern medicine:identification of therapeutic targets for suppression of inflammation and cancer". Expert Opin. Ther. Targets (2006) 10(1)

[16] VR Ahire, KP Mishra, G. K. (2015). "Apoptotic Radiosensitivity of Cervical Tumor Cells Enhanced by Ellagic Acid". EJBB.2015. 3(4) 56-58

[17] S.Girdhani , SM Bhosle, SA Thulsidas, AKumar, KP Mishra . "Potential of radiosensitizing agents in cancer chemo-radiotherapy". Journal of Cancer Research and Therapeutics, 2005.1(3), 129.

[18] VR Ahire, KP Mishra, GR Kulkarni. "On the mechanism of cellular toxicity in breast cancer by ionizing radiation and chemotherapeutic drugs". JEPTO, 2014.33(1), 69–82.

[19] SM Bhosle, VR Ahire, MS Henry, VS Thakur, NG Huilgol, KP Mishra. "Augmentation of radiation-induced apoptosis by ellagic acid". Cancer Investigation, 2010. 28(3), 323–30.

[20] B Kumar, A Kumar, BN Pandey, BHazra, KP Mishra. "Increased cytotoxicity by the combination of radiation and diospyrin diethylether in fibrosarcoma in culture and in tumor". International Journal of Radiation Biology, 2008. 84(5), 429-40.